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Characterization of an In Vitro System for the Synthesis of mRNA from Human Parainfluenza Virus Type 3

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A cell extract derived from human parainfluenza virus type 3-infected human lung carcinoma (HLC) cells synthesized mRNA in vitro. Under optimal conditions, the extract was able to support transcription of all virus-encoded genes as determined by hybridization analyses. The RNA products contained full-length poly(A)-containing mRNA species similar to those observed in acutely infected cells. Further purification of the viral nucleocapsids from the infected HLC cell extract resulted in total loss of the capacity of the extract to synthesize mRNA in vitro. However, the addition of cytoplasmic extracts from uninfected HLC cells to the nucleocapsid preparations restored transcription to levels observed in the infected cell lysates, indicating requirement of a host factor(s) in the human parainfluenza virus type 3 transcription process. In distinction to the abundant transcription observed in the cell extract from HLC cells, cell extract prepared from CV-1 cells failed to support transcription in vitro. High levels of RNase activity in the cell extract from CV-1 cells appears to be the principal reason for this difference.

The *Paramyxoviridae* are enveloped, negative-strand, nonsegmented RNA viruses. The three genera grouped within this family include the *Morbillivirus* (measles, canine distemper, and rinderpest viruses), *Paramyxovirus* (Newcastle disease, mumps, and human parainfluenza type 1 to 5 viruses), and the *Pneumovirus* (respiratory syncytial and mouse pneumonia viruses) groups. Human parainfluenza virus type 3 (HPIV-3) is second in importance only to respiratory syncytial virus as a serious lower respiratory tract pathogen in early childhood (10, 11, 23). The genome of HPIV-3 is a single-stranded negative-sense RNA of 15,461 nucleotides contained within a helical nucleocapsid (20). Based on the nucleotide sequence analysis of the genes, the amino acid sequences for all the structural proteins are known (13–20, 27, 29, 30, 38, 41–46). Six distinct structural proteins have been identified in purified virions and in infected cells; these include an RNA-dependent RNA polymerase L (251 kilodaltons [kDa]), a phosphoprotein, P (90 kDa), a nucleocapsid protein, NP (68 kDa), a hemagglutinin-neuraminidase protein, HN (71 kDa), a fusion protein, F_0 (65 kDa), and a matrix protein, M (35 kDa). In addition to the virion-associated proteins, a nonstructural protein, C (23 kDa), is encoded within the P mRNA in an overlapping reading frame (26, 30, 40, 43, 47, 49). Yet another nonstructural protein (V protein) has been detected in several paramyxovirus-infected cells, again arising from the P gene (8, 36, 48).

Although considerable knowledge has been gathered on the molecular organization of HPIV-3, the mechanisms involved in transcription and replication of this virus remain undefined primarily owing to lack of an efficient in vitro transcription system. The purified ribonucleoprotein (RNP) complexes of HPIV-3 consist of the single-stranded genomic RNA encapsidated with the NP protein. Associated with these complexes are the P and L proteins. By analogy with other paramyxoviruses and rhabdoviruses, these proteins are thought to play important roles in the synthesis, capping, methylation, and polyadenylation of mRNA species. The precise role of these three proteins in the above functions has not yet been established. Recently, requirements for host cell proteins as positive transcription factors for in vitro RNA synthesis by negative-strand RNA viruses have been reported (35).

In an effort to study in detail the molecular mechanism of gene expression of HPIV-3 and to elucidate the roles of different proteins in that process, we developed an in vitro transcription system for this virus. The cell-free transcription system supported the synthesis of all virus-encoded HPIV-3 mRNA species. In addition, involvement of a host factor(s) in this process was also documented.

MATERIALS AND METHODS

Cells and virus. CV-1 cells were propagated as monolayers in Dulbecco modified Eagle medium supplemented with 5% fetal bovine serum, penicillin (50 U/ml), and streptomycin (50 μg/ml). Human lung carcinoma (HLC) cells (ATCC CCL 185, A549) were propagated as monolayers in Eagle minimum essential medium supplemented with 5% fetal bovine serum, penicillin (50 U/ml), and streptomycin (50 μg/ml).

HPIV-3 (HA-1; NIH 47885) was grown in CV-1 cells. The cells were infected with the virus at a multiplicity of infection of 0.05 PFU per cell in 10 ml of serum-free medium. After 2 h of incubation at 37°C for viral adsorption, the medium was removed and 50 ml of fresh medium containing 5% fetal bovine serum was added. At 48 h postinfection, virus was released by freeze-thawing. Cell debris were removed by centrifugation at 1,500 × *g* for 10 min. The supernatant was then centrifuged at 35,000 rpm for 1 h at 4°C in a Beckman 70 Ti rotor. The virus pellet was suspended in 20 mM Tris hydrochloride (pH 7.5) and stored in liquid nitrogen.

Preparation of cell extract. Monolayers of CV-1 and HLC cells (3×10^7 cells in a 100-mm dish) were infected with HPIV-3 at 5 PFU per cell in 1 ml of serum free Eagle minimum essential medium. At 2 h postinfection, 10 ml of fresh minimum essential medium containing 5% fetal bovine serum was added and incubated at 33°C for an additional 22

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h. Cell extract was prepared from HPIV-3-infected and mock-infected cells essentially as described by Peluso and Moyer (37) and subsequently modified by Carlsen et al. (7). Briefly, the cells were washed with phosphate-buffered saline and were then treated with lysolecithin (250 μg/ml) at 4°C for 1 min. Following this treatment, the cells were scraped into 100 μl of lysis buffer containing 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) adjusted to pH 8.0 with KOH, 50 mM NH_4Cl, 7 mM KCl, 4.5 mM magnesium acetate, 1 mM dithiothreitol, 1 mM each ATP, GTP, and UTP, 10 μM CTP, 2 μg of actinomycin D per ml, 40 U of creatine kinase per ml, 50 mM creatine phosphate, and 1 mM spermidine. The cells were disrupted by pipetting 15 times with a Pasteur pipette, and the lysate was centrifuged at 800 × *g* for 5 min to remove nuclei and cell debris. The supernatant was collected, and the volume was adjusted to 150 μl with the lysis buffer. The protein concentration was estimated as 6.5 mg/ml by using the Bio-Rad protein assay kit. Cell extract from CV-1 cells was prepared by the identical procedure, and the protein concentration in the lysate was 5.0 mg/ml. The resulting cell extracts were stored at −70°C for subsequent use in transcription reaction.

Purification of RNP complexes from cell extracts. RNP complexes were isolated from the cell extract from HPIV-3-infected HLC cells by centrifugation at 40,000 rpm for 2 h at 4°C through 40% glycerol in 20 mM HEPES-KOH (pH 7.5)–1 mM dithiothreitol (200 μl) onto a 100% glycerol cushion (50 μl), using 0.8-ml-capacity tubes with an adapter in an SW50.1 rotor. The RNP complexes, pelleted on the top of 100% glycerol, and the soluble proteins, remaining at the top of 40% glycerol, were collected separately and stored at −70°C. Soluble cytoplasmic proteins from uninfected cells were prepared in the same way. Protein concentrations in the RNP fraction and in the soluble fraction were estimated as 7.5 and 5.0 mg/ml, respectively.

In vitro RNA synthesis and product analysis. An in vitro transcription reaction was done in a 100-μl reaction volume containing the lysis buffer with the NH_4Cl concentration adjusted to 0.15 M, 40 μCi of [α-^{32}P]CTP, and 25 μl of cell extract or 7 μl of RNP complex plus 20 μl of soluble cytoplasmic proteins from uninfected cells. Incubation was performed at 30°C for 3 h. At the end of the reaction, a 2-μl sample was used for monitoring RNA synthesis by the DE-81 paper binding assay (5). In parallel, the transcription reaction was done with cell extract from mock-infected cells. Virus specific RNA synthesis was determined by subtracting the synthesis obtained in the mock-infected cell extract from that obtained in the HPIV-3-infected cell extract.

^{32}P-labeled RNA products were separated from unincorporated radioisotope in a spun column (31). The purified RNA products were then analyzed in 1.8% agarose gel after glyoxal denaturation (34)

Protein analysis. Cell extracts were prepared as described above at 24 h postinfection from HPIV-3-infected HLC cells labeled with [^{35}S]methionine (50 μCi/ml) in serum-free Eagle minimum essential medium from 18 to 24 h postinfection. To prepare radiolabeled virus, we labeled HPIV-3-infected CV-1 cells with [^{35}S]methionine (10 μCi/ml) at 10 h postinfection. The supernatant fluid was harvested at 48 h postinfection, and virus was concentrated by centrifugation at 35,000 rpm for 1 h in a 70 Ti rotor. The viral pellet was suspended in 20 mM Tris hydrochloride (pH 7.5). Viral proteins were immunoprecipitated by the method of Bernstein and Hruska (6). The cell extract or the RNP complex was incubated with anti-HPIV-3 serum in RIPA buffer for 16 h at 4°C. The antigen-antibody complexes were collected by binding to *Staphylococcus aureus* and eluted by boiling for 5 min in polyacrylamide gel loading buffer. The bacteria were removed by centrifugation, and the proteins were analyzed by electrophoresis in a sodium dodecyl sulfate (SDS)-polyacrylamide gel (28). The gel was processed for fluorography (9) and exposed to an X-ray film at −70°C.

TABLE 1. Synthesis of HPIV-3-specific RNAs in vitro in cell extracts from HLC and CV-1 cells[a]

Cell lysate	Infection	[^{32}P]CMP incorporation (10^4 cpm/3 h)
HLC	Mock	35
	HPIV-3	115
CV-1	Mock	4
	HPIV-3	6

[a] Reaction conditions are as described in Materials and Methods.

Slot-blot analysis. HPIV-3 cDNA clones NP, P, M, F, HN, and L (16–20) and two other unrelated cDNA clones, NS and N of vesicular stomatitis virus strain NJ (22, 33), were used in slot-blot analysis. The purified plasmid DNAs were digested with appropriate restriction endonucleases to release the insert cDNAs. The digested DNA (2 μg) was denatured in 0.2 M NaOH, applied to Gene Screen membrane with the Bio-Rad slot-blot apparatus, and hybridized to in vitro-synthesized radiolabeled RNA products according to the protocol of the manufacturer. A reaction mixture similar to the one described above was used to prepare ^{32}P-labeled in vitro RNA transcripts, except that 100 μCi of [α-^{32}P]CTP was used in a 200-μl reaction volume. Hybridization was done at 42°C for 36 h, and the membrane was washed sequentially with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature, 2× SSC plus 1% SDS at 65°C, and 0.1× SSC at room temperature. The blot was autoradiographed at −70°C with an intensifying screen. Densitometric scanning of the autoradiogram was done in a Bio-Rad laser densitometer scanner.

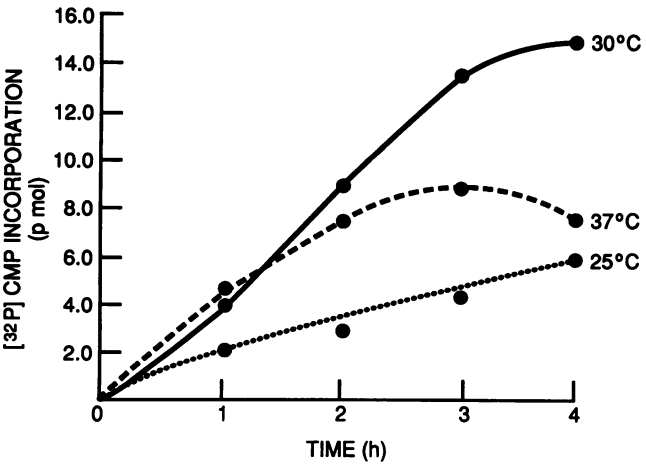

FIG. 1. Time course of RNA synthesis. Transcription reaction mixtures containing cell extract (25 μl) from HPIV-3-infected HLC cells were incubated at 25, 30, and 37°C for the indicated times. Similar transcription reactions were done with mock-infected cell extract. RNA synthesis was monitored by a DE-81 binding assay, and virus-specific RNA synthesis in a 100-μl reaction mixture was obtained by subtracting the incorporation of [^{32}P]CMP in mock-infected cell lysate from that obtained in the infected cell lysate.

TABLE 2. Requirements for HPIV-3 transcription

Exptl conditions for RNA synthesis	[^{32}P]CMP incorporation (pmol)[a]
Cell lysate (μl)	
10	6.3
20	13.8
40	17.6
NH_4Cl (mM)	
50	3.0
100	11.42
150	14.54
Mg^{2+} (mM)	
2	5.4
4	14.6
6	12.4
pH	
7.5	7.37
8.0	16.90
8.5	12.46

[a] Incorporation of [^{32}P]CMP in 3 h in a 100-μl reaction mixture. Reaction conditions are as described in Materials and Methods.

RESULTS

In vitro synthesis of HPIV-3 RNAs by infected HLC cell extract. To develop an in vitro system for the synthesis of mRNAs from HPIV-3, we used two different cell lines, CV-1 and HLC, and studied their ability to support transcription of this virus in a cell-free system. Since in both the cell lines no detectable virus release occurred at 24 h postinfection, that time point was selected for the preparation of cell extract from HPIV-3-infected cells. Incorporation of [α-^{32}P]CTP into HPIV-3-specific RNA products in these two cell-free systems was measured by a DE-81 binding assay. Virus-specific RNA synthesis occurred in the cell extract from HLC cells, whereas no significant RNA synthesis was detected in similar cell extract derived from CV-1 cells (Table 1). Thus, HLC cells were chosen for the studies reported here; the apparent inability of CV-1 cell extract to support transcription in vitro is due to the presence of RNase in the extract (see below).

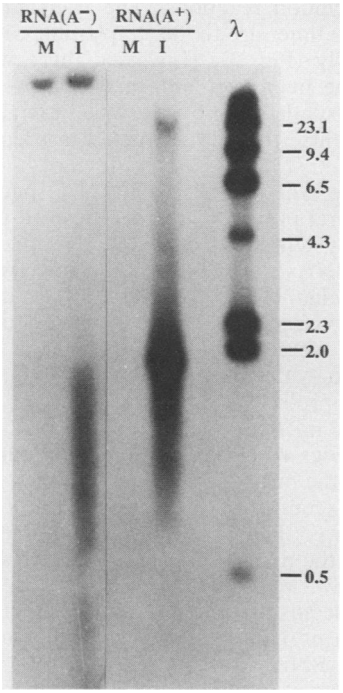

FIG. 2. Electrophoretic analysis of RNA products. ^{32}P-labeled in vitro-synthesized RNAs were separated into poly(A)$^+$ and poly(A)$^-$ species by oligo(dT)-cellulose column chromatography. RNA was denatured by treatment with glyoxal-dimethyl sulfoxide and analyzed by electrophoresis on a 1.8% agarose gel. Poly(A)$^+$ and poly(A)$^-$ RNAs synthesized in mock-infected (M) and infected (I) cell extracts are shown. Lambda DNA digested with *Hin*dIII was heated for 5 min at 100°C with 50% formamide, chilled on ice, and loaded on the gel as size markers.

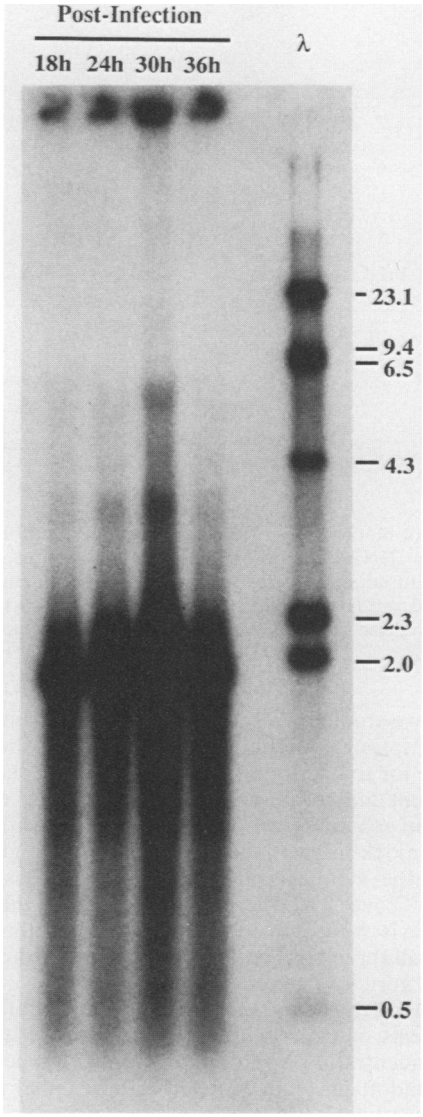

FIG. 3. RNA synthesis by cell extracts harvested at different times postinfection. ^{32}P-labeled RNAs that were synthesized in vitro with extracts (25 μl) harvested at various times postinfection as indicated were analyzed in a 1.8% agarose gel upon denaturation with glyoxal-dimethyl sulfoxide. The gel was dried and autoradiographed at −70°C with X-AR film. Lambda DNA digested with *Hin*dIII was used as size markers (in kilobases).

Determination of optimal conditions for in vitro transcrip-

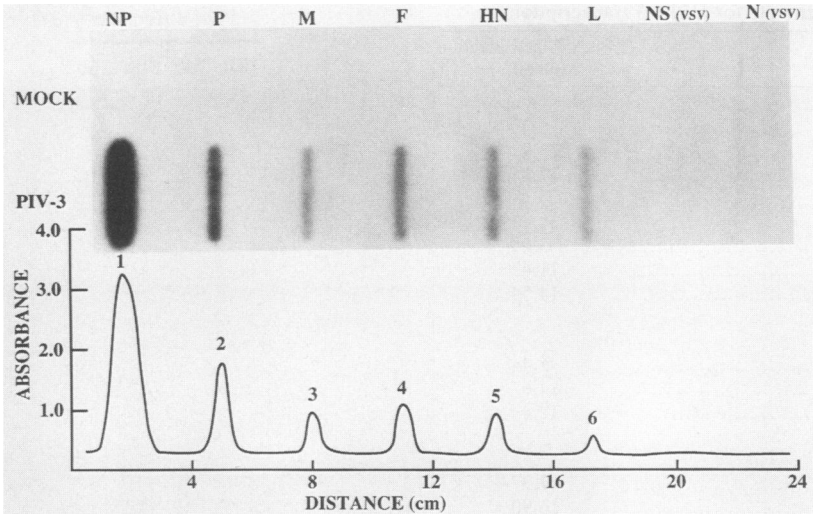

FIG. 4. Slot-blot analysis of RNA products. Plasmid DNAs (2 μg each) prepared from NP, P, M, F, HN, and L cDNA clones of HPIV-3 and NS and N cDNA clones of vesicular stomatitis virus strain NJ (VSV_{NJ}) were digested with restriction endonucleases to release the insert cDNAs, denatured, and blotted onto a Gene Screen membrane. ^{32}P-labeled RNAs synthesized in vitro in transcription reactions containing mock-infected and HPIV-3-infected cell extracts were used as probes. The blots were hybridized and washed as described in Materials and Methods. The dried blot was then subjected to autoradiography. Densitometric scanning of the autoradiogram was done in a Bio-Rad laser densitometer.

tion. Next, we studied several parameters to optimize the in vitro transcription condition. To characterize the optimal temperature for transcription, we performed the reactions at three different temperatures, 25, 30, and 37°C, over 4 h. Transcription reactions were done in parallel with cell extract from mock-infected cells. A linear, time-dependent increase in the synthesis of virus-specific RNAs was observed at 30°C over 3 h, whereas at 25 and 37°C, the levels of RNA synthesis were considerably lower than that at 30°C (Fig. 1). At all three temperatures, mock-infected cell lysate synthesized very low levels of RNA products.

The in vitro RNA synthesis depended on viral proteins, since synthesis of RNA increased almost linearly with increasing concentration of protein from infected cells (Table 2). NH_4Cl and magnesium acetate requirements for optimal transcription were 150 and 4 mM, respectively (Table 2). Effect of pH on viral transcription over a range of 7.5 to 8.5 was studied, and the optimum pH was found to be 8.0 (Table 2).

Analysis of RNA products synthesized in vitro. The in vitro RNA products synthesized under optimal condition were selected for poly(A)$^+$ RNAs by passage through an oligo(dT)-cellulose column (3). The bound and unbound RNAs were analyzed in an agarose gel after denaturation with glyoxal. Poly(A)$^+$ RNA transcripts ranging in size from 0.8 to 2.6 kilobases (kb) were synthesized (Fig. 2). On the other hand, oligo(dT)-unbound RNA species, representing only 5% of the total RNA, migrated as a diffuse band smaller than 2 kb. Since the sizes of the six mRNAs encoding HPIV-3 structural and nonstructural proteins have been reported to be 1.64 kb (NP), 2.02 kb (P), 1.15 kb (M), 1.85 kb (F), 1.89 kb (HN), and 6.79 kb (L) (13–20, 27, 29, 30, 38, 41–46), it seems that the majority (95%) of the in vitro-synthesized mRNAs contain all mRNA species, possibly with the exception of the L mRNA, and are full length and poly(A)$^+$. A major band migrating slower than 2 kb probably represents the NP mRNA. No other discrete band was apparent, possibly owing to closeness of mRNA sizes and heterogeneity in poly(A) tail length.

To study whether discrete mRNA species, larger than observed, can be synthesized by the extract, we prepared cell extracts from HPIV-3-infected HLC cells harvested at different postinfection periods and the extracts were then used in transcription reactions. The transcriptase activity increased with time up to 30 h postinfection and then decreased slowly (Fig. 3). However, there was no major difference in the migration pattern in agarose gel of these RNA products synthesized by these cell extracts. In subsequent experiments, the cell extract was prepared at 30 h postinfection.

To determine whether all the mRNA species encoding HPIV-3 structural proteins were synthesized in the in vitro transcription system, we did slot hybridization. Equal amounts of cDNAs derived from the mRNAs of HPIV-3 structural proteins NP, P, M, F, HN, and L and two unrelated cDNAs such as NS and N of vesicular stomatitis virus strain NJ were immobilized on a Gene Screen membrane. In vitro-synthesized ^{32}P-labeled RNA transcripts were used as probes in the hybridization reaction. All HPIV-3-specific mRNAs, including L mRNA, were synthesized in vitro (Fig. 4). Note that there is a gradual decrease of synthesis of the mRNAs in the order NP > P > F > HN > L, with the exception of the M protein mRNA, which was consistently lower than F mRNA. Densitometric scanning showed that of the total transcripts, NP mRNA represented 49.3%; P, 15.6%; M, 8.2%; F, 10.3%; HN, 9.5%; and L, 7.0%. Thus, it seems that there is a polar effect on transcription in vitro as previously shown for other nonsegmented negative-strand RNA viruses (1, 12, 21, 24). However the reason for inadequate synthesis of M protein mRNA in vitro is not clear.

Analysis of viral proteins in cell extract. To study which virus-specific proteins are present in the cell extract, [^{35}S]methionine-labeled proteins in cell extracts derived from HPIV-3-infected and mock-infected HLC cells were analyzed directly (Fig. 5, Total) or after immunoprecipitation (Fig. 5, Immune ppt) with anti-HPIV-3 serum in an SDS-10% polyacrylamide gel. The viral nucleocapsid pro-

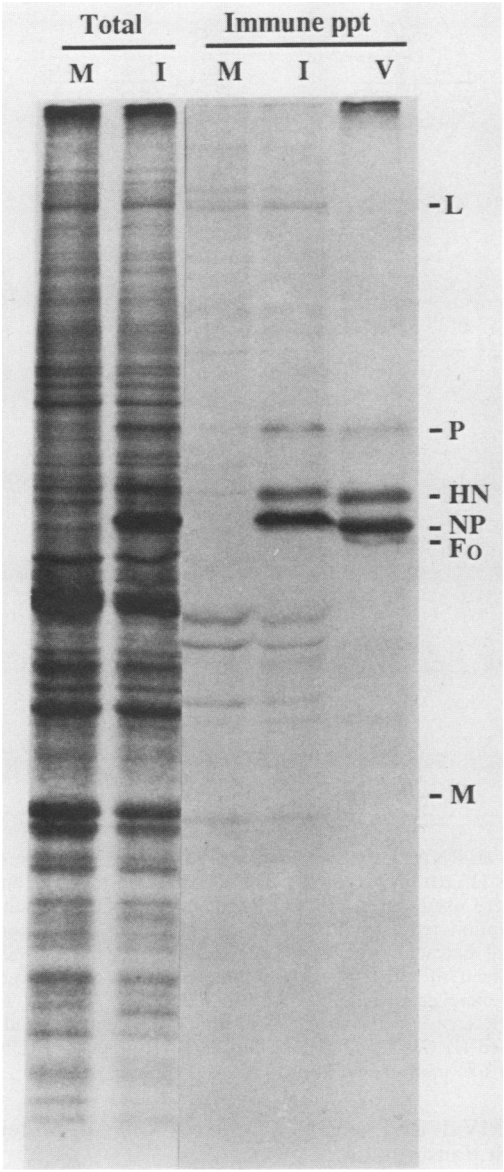

FIG. 5. Analysis of viral proteins in cell extract. Extracts of HLC cells mock infected (M) or infected (I) with HPIV-3 and labeled with [^{35}S]methionine (25 μCi/ml) were analyzed directly or after immunoprecipitation (ppt) with anti-HPIV-3 serum in an SDS-10% polyacrylamide gel. The gel was fluorographed, dried, and exposed to X-AR film with an intensifying screen at −70°C. Lane V, Immunoprecipitated proteins from purified virus. Migration positions of the viral proteins are shown on the right.

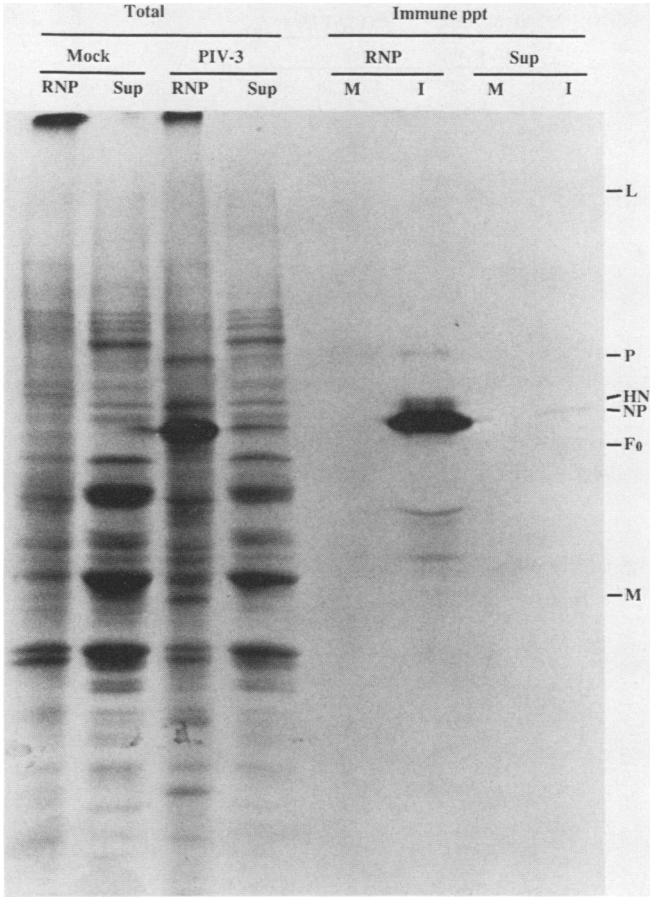

FIG. 6. Analysis of viral proteins in the purified RNP. Mock-infected and HPIV-3-infected HLC cell proteins were labeled with [^{35}S]methionine (25 μCi/ml). Cell extract was prepared as described in Materials and Methods. RNP was purified from the cell extract by centrifugation through 40% glycerol for 2 h at 40,000 rpm with an SW 50.1 rotor in a Beckman ultracentrifuge. Radiolabeled proteins present in the RNP fraction and in the soluble fraction were analyzed directly or after immunoprecipitation (ppt) with anti-HPIV-3 serum in an SDS-10% polyacrylamide gel. The gel was fluorographed, dried, and exposed to X-AR film at −70°C with an intensifying screen. The fluorogram shows the total proteins in the RNP fraction and in the soluble fraction (Sup) of mock-infected and HPIV-3-infected cells and the immunoprecipitated proteins from the RNP fraction of mock-infected (M) and HPIV-3-infected (I) cells. Migration positions of the viral proteins are shown at the right.

teins NP and P and the HN glycoprotein were discernible over the background of host cell proteins. All these proteins were precipitated by anti-HPIV-3 serum, indicating that these proteins are of viral origin. The L protein, on the other hand, was not readily distinguishable from the host proteins because of its presence in low amounts and also because of the migration of several cellular proteins in the same position as L in the SDS-polyacrylamide gel. Some minor cellular protein bands were also present in the immunoprecipitate, possibly owing to nonspecific binding with the antibody.

Involvement of cellular components in HPIV-3 transcription. In an attempt to further purify HPIV-3 RNP complexes from infected HLC cells free from cellular proteins, the cell lysate was centrifuged through 40% glycerol and the RNP complex was collected on a 100% glycerol cushion. To examine the viral proteins in the pellet, we analyzed [^{35}S]methionine-labeled proteins on an SDS-10% polyacrylamide gel (Fig. 6). The RNP complex contained NP, P, HN, and, in addition, a small amount of cellular proteins. The presence of NP and P proteins was further confirmed by immunoprecipitation with anti-HPIV-3 serum. Again, the L protein was not easily discernible in the immune complex, possibly due to its presence in a low amount in the infected cells together with weak interaction with antibody which was raised against the whole virus. Surprisingly, when transcription was performed with these RNP complexes, very little transcript of the full-length mRNA size class was synthesized (Fig. 7). However, the transcriptase activity was fully

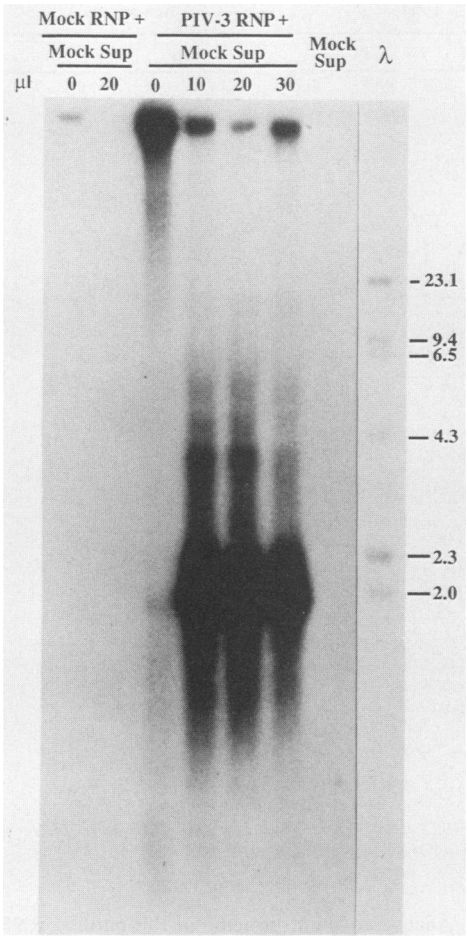

FIG. 7. Effect of soluble proteins from uninfected cells on RNA synthesis by purified RNP. RNP was purified from cell extract that was prepared from HPIV-3-infected HLC cells as described in Materials and Methods. The purified RNP (7 μl) was then used in the transcription reaction. The effect of soluble proteins from uninfected cells on the transcription reaction was examined by adding them to the RNP and then performing the transcription reaction. RNA products were purified, denatured in glyoxal-dimethyl sulfoxide, and analyzed in a 1.8% agarose gel. RNAs synthesized in vitro by RNP from mock-infected cells, by RNP from mock-infected cells plus mock-infected cell soluble proteins (Mock Sup), by HPIV-3-infected cells RNP, and by HPIV-3-infected cell RNP plus mock-infected cell soluble proteins (Mock Sup, 10, 20, and 30 μl) are shown. RNA synthesized in vitro by mock-infected cell soluble proteins alone is also shown. *Hin*dIII-digested λ DNA was used as size markers.

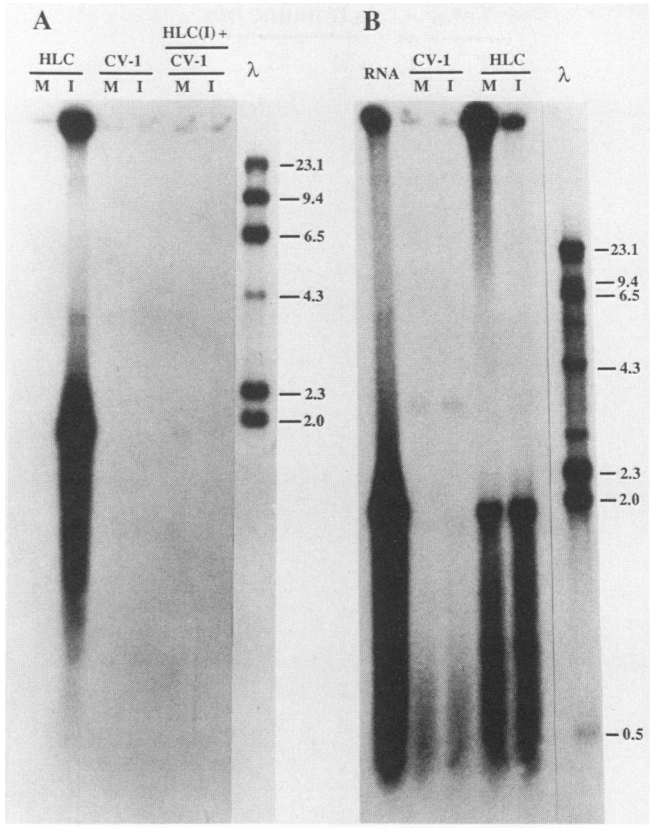

FIG. 8. RNA synthesis by cell extract from CV-1 cells. (A) Cell extracts (15 μl) prepared from HPIV-3-infected (I) HLC and CV-1 cells were used separately and in combination (15 μl each) in the transcription reaction. Similar transcription reactions were done with cell extracts from mock-infected (M) cells. (B) The RNAs synthesized in vitro in HPIV-3-infected HLC cell extract were purified, and equal amounts were incubated at 30°C for 1 h with buffer (RNA) or cell extracts from mock-infected (M) and HPIV-3-infected (I) CV-1 and HLC cells. *Hin*dIII-digested λ DNA was used as size markers (in kilobases).

restored upon the addition of soluble cytoplasmic proteins from mock-infected HLC cells to the RNP complex. These results indicate that the inability of the RNP complex to transcribe was not due to the apparent lack of the L protein associated with it. Neither soluble proteins (Fig. 7, Mock Sup) or high-speed pellet (Fig. 7, Mock RNP) synthesized any detectable RNA in vitro. An approximately 10-fold stimulation of transcription was obtained by the addition of 10 μl (65 μg) to 20 μl (130 μg) of soluble proteins. No further stimulation was observed with addition of greater amounts of cellular proteins. The stimulatory activity of the mock-infected cell proteins was completely abolished when they were heated to 65°C for 2 min before use in the transcription reaction (data not shown). These results clearly indicate that the HPIV-3 RNP complex requires cellular proteins for efficient transcription.

RNA synthesis in vitro by cell extract from infected CV-1 cells. Finally, to study the reasons for the apparent inability of the cell extract prepared from HPIV-3-infected CV-1 cells to direct RNA synthesis, we examined for the presence of any inhibitory component or RNase activity in the CV-1 cell extract. Transcription reactions were performed separately with cell extracts from HPIV-3-infected and mock-infected HLC and CV-1 cells. The reaction products were analyzed in a 1.8% agarose gel after glyoxal denaturation (Fig. 8). Unlike HLC cell extract, there was no synthesis of HPIV-3 transcripts in CV-1 cell extract. To study the effect of CV-1 cell extracts on HPIV-3-specific RNA synthesis, we added CV-1 cell extracts to the transcribing HLC cell extract. RNA synthesis was virtually abolished by both mock-infected and infected CV-1 cell extracts. Next, we examined for the presence of RNase activity in the extract. Purified transcripts obtained from an in vitro transcription reaction were incubated with mock-infected and HPIV-3-infected cell extracts of these cell lines. In both cases, the transcripts were completely degraded. On the other hand, in HLC cell extract, 50% of the transcripts were still protected. This suggests that CV-1 cells contain a high level of RNase

activity, and this may be the reason for the inability of this cell extract to demonstrate RNA synthesis in vitro.

DISCUSSION

We developed an in vitro transcription system for HPIV-3 to study the mechanism of gene expression of this important human pathogen. The results presented here demonstrate that RNP isolated from HPIV-3-infected HLC cells efficiently synthesized RNA in vitro. The RNA synthesis was not dependent on de novo viral protein synthesis, as in bunyaviruses (2, 4), since the addition of cycloheximide (up to 100 μg/ml) did not inhibit transcription in vitro (data not shown). By slot-blot hybridization with cDNA from six HPIV-3 mRNA species, it was shown that all mRNAs species are synthesized in vitro (Fig. 4). The mRNAs were polyadenylated (Fig. 2) and full length as analyzed by Northern (RNA) blot hybridization (data not shown). By direct analysis of the in vitro-synthesized RNAs in a 20% polyacrylamide-urea gel, we were unable to detect synthesis of 55-nucleotide-long leader RNA (data not shown). This may be due to specific degradation of uncapped leader RNA by exonucleases present in the cell extract (32). Optimal transcription occurred at 30°C in the presence of 150 mM NH_4Cl and 4 mM magnesium acetate at pH 8.0 and with RNP isolated from the infected cells 30 h postinfection. Using similar extraction and transcription conditions, RNP isolated from cells infected with measles virus (39) and respiratory syncytial virus (25) has recently been shown to synthesize virus-specific mRNAs.

By quantitation by slot-blot analyses of the level of HPIV-3 mRNAs synthesized in vitro (Fig. 4), a distinct polar effect on transcription was observed—NP mRNA was the most abundant species, while L mRNA was the least abundant. These results are consistent with the observed sequential transcription of the genome RNA of nonsegmented negative-strand viruses (1, 12, 21, 24) as measured by UV transcription mapping experiments. However, we have consistently observed that M protein mRNA synthesis was less than that of its 5′-proximal F gene (Fig. 4). The reason for this anomalous synthesis of the M mRNA is not presently clear. It is unlikely that the M mRNA is specifically degraded in the transcription reaction. The polymerase, on the other hand, may transcribe the M gene at a lower frequency, or there is an independent internal initiation beginning at the start of the F gene. Interestingly, for measles virus in vitro transcription, the amount of hemagglutinin mRNA was found to be higher than that of its 3′-proximal F gene transcript (39). Further studies are needed to gain insight into this phenomenon.

Upon further purification of the transcribing cell extract containing HPIV-3 RNP through 40% glycerol, a pellet containing NP, P, and L proteins was obtained which completely lacked the capacity to transcribe RNA in vitro (Fig. 7). However, the addition of soluble protein from the cytoplasmic fraction of uninfected cells fully restored the transcriptive potential of the RNP (Fig. 7). The activation property of the host cell extract was completely abolished by heating at 65°C for 2 min, indicating that the activating component is a protein.

These results clearly demonstrate that host protein(s) plays an important role in the HPIV-3 transcriptive process. It is presently unclear, however, whether the putative host protein(s) is absolutely needed for transcription or compensating for the loss of a viral component in vitro. Moyer et al. (35) have recently demonstrated that host protein(s) is required for transcription of purified Sendai virus in vitro. In addition, they showed that one of the host proteins was tubulin since the addition of tubulin stimulated transcription and antitubulin antibody inhibited the in vitro transcription reaction (35). Our results similarly indicated that cellular proteins are required for HPIV-3 transcription, although we did not determine whether tubulin stimulates transcription in the HPIV-3 system. Detergent-disrupted HPIV-3 purified from HLC cells did not synthesize RNA in vitro and was not activated by cellular protein(s) (data not shown). This may be due to an insufficient amount of purified virus used or to low transcriptase activity in purified HPIV-3. We are currently fractionating the soluble cytoplasmic fraction of mock-infected cells to isolate and characterize the putative cellular activating component.

Finally, isolation of the active transcription complex of HPIV-3 depended principally on the cells from which the extract was prepared. The RNP fraction purified from a cell line highly permissive for HPIV-3, such as CV-1, failed to synthesize RNA although it contained NP, L, and P proteins in adequate quantities (Table 1). This was solely due to the presence of a high level of RNase activity in the extract (Fig. 8). In contrast, HLC cells either contained low levels of RNase activity or the RNP fraction somehow did not associate strongly with RNase during the isolation procedure. Purification of RNP complex free of RNase from HPIV-3-infected CV-1 cells is currently in progress. The development of an active in vitro transcription system of HPIV-3 would certainly help elucidate the mechanism of transcription and replication processes of the virus as well as functions of the polypeptides in the transcription complex and the role of host factor in these processes.

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